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MULTIPLE VARIANTS OF MYOFIBRILLAR PROTEINS IN SINGLE FIBERS OF LOBSTER CLAW MUSCLES: EVIDENCE FOR TWO TYPES OF SLOW FIBERS IN THE CUTTER CLOSER MUSCLE[1]

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ABSTRACT

SDS-polyacrylamide gel electrophoresis of myofibrillar proteins in single fibers of lobster claw closer muscles distinguished three types of fibers: one fast and two slow. The fibers differed both qualitatively and quantitatively in the variants of paramyosin and troponin present. There were four proteins unique to fast fibers (paramyosin$_1$, a 75-kD protein, troponin-I_3, and -I_5) and two proteins unique to slow fibers (troponin-I_4 and -C_1). Fast fibers were found only in the cutter claw. The major type of slow fibers (S_1) appeared to account for the entire muscle mass in the crusher claw as well as ~85% of the slow fibers in the cutter claw. Another type (S_2) comprised 10–15% of the slow fibers in the cutter claw. The S_1 and S_2 fibers differed in the variants of troponin-I and -T. The S_2 fibers contained troponin-T_1 and I_2 as the major variant of troponin-I; S_1 fibers lacked T_1 and contained I_4 as the major isoform. These data indicate that the heterogeneity of myofibrillar proteins observed in actomyosins extracted from whole muscle (Mykles, 1985) is due to three populations of fibers, each containing its own assemblage of regulatory and contractile isoforms. More than one variant of a myofibrillar protein can be expressed in a single fiber, forming unique assemblages by which subgroups can be discriminated within the broader categories of fast and slow fibers.

INTRODUCTION

Physiological, morphological, and histochemical methods have been used to classify crustacean muscle fibers. Fast, or phasic, fibers have short sarcomeres, low ratios of thin:thick myofilaments, fast contraction speeds, low oxidative capacities, and high ATPase activities. Conversely, slow, or tonic, fibers have longer sarcomeres, higher ratios of thin:thick myofilaments, slower contraction speeds, higher oxidative capacities, and lower ATPase activities (Jahromi and Atwood, 1969, 1971; Lang *et al.,* 1977, 1980; Mykles and Skinner, 1981; Stephens *et al.,* 1984; Mykles, 1985). Fibers with intermediate properties have also been described (Jahromi and Atwood, 1971; see review by Govind and Atwood, 1982). Occasionally these methods can discriminate between fiber subgroups (Kent and Govind, 1981; Parsons and Mosse, 1982). More often characteristics defined by such methods do not delineate absolute differences between fast, slow, and intermediate fibers within a species; comparisons between species are even less definitive. SDS-polyacrylamide gel electrophoresis is a method that should serve to more rigorously distinguish muscle fiber types in Crustacea.

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[1] Abbreviations used: ATP, adenosine triphosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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Heterogeneity of myofibrillar proteins has been described recently in the muscles of two crustacean species, the lobster, *Homarus americanus* (Costello and Govind, 1984; Mykles, 1985), and snapping shrimp, *Alpheus heterochelis* (Quigley and Mellon, 1984). Moreover, actomyosins extracted from lobster muscles contain multiple variants of regulatory and contractile proteins (Mykles, 1985). These isoforms of myofibrillar proteins comprise four distinct assemblages in fast and slow muscles. A total of two variants of paramyosin, three of troponin-T, five of troponin-I, three of troponin-C, three of myosin alpha light chain (αLC) and two of myosin beta light chain (βLC) are found in six muscles of the claws and abdomen (Mykles, 1985). Myosin heavy chain, actin, and tropomyosin appear isomorphic in all lobster muscles examined. The closer muscles of the cutter and crusher claws differ primarily in the species of paramyosin and troponin-I and -C; two or more variants of these proteins can occur in a single muscle. This heterogeneity may be caused by mixtures of different fibers, each having particular protein variants, or by a homogeneous population of fibers that contain a mixture of protein variants in a proportion characteristic of the specific muscle. To distinguish between these two alternatives, individual fibers from the claw closer muscles of adult lobsters were glycerinated and analyzed by SDS-PAGE which, coupled with silver staining, is sufficiently sensitive to detect proteins from single fibers. The data show three distinct fiber types, rather than the two types classically defined (see Govind and Atwood, 1982, for references). The slow fibers of the crusher claw and

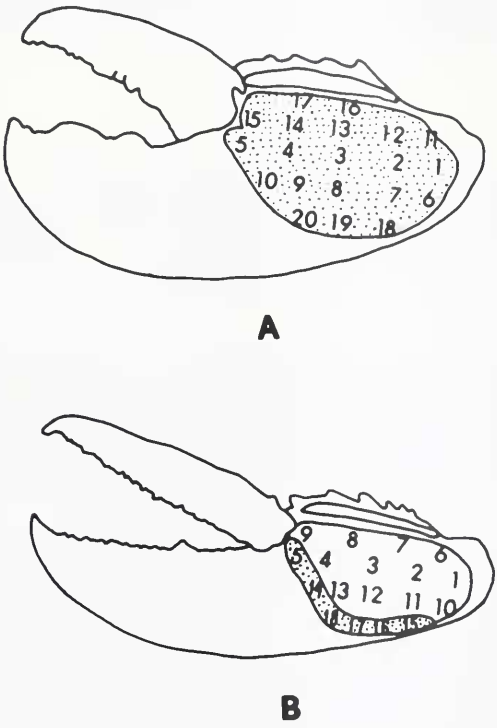
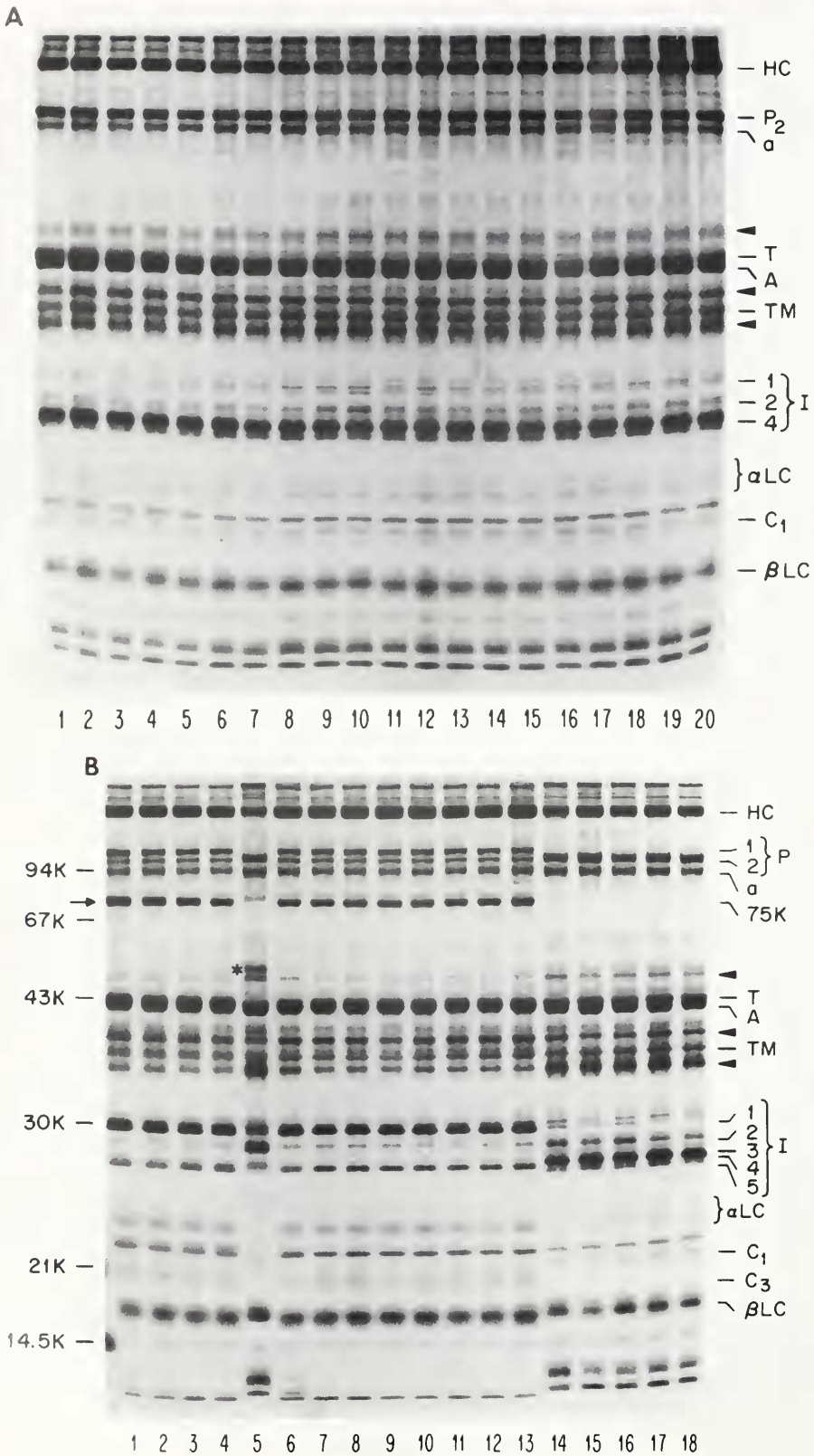


FIGURE 1. Diagram of lobster crusher (A) and cutter (B) claws showing locations from which single fibers were removed for analysis by SDS-PAGE. Numbers correspond to lane numbers in Figure 2. Stippling indicates areas containing slow fibers; fast fibers were located in the dorsal and proximal regions of the cutter closer muscle (see Lang *et al.*, 1980).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the fast fibers of the cutter claw consisted of homogeneous populations. By contrast, there were two types of slow fibers in the cutter claw, each containing a different assemblage of troponin variants.

Materials and Methods

Homarus americanus (0.6–0.9 kg) were obtained locally. Claws were removed and placed on ice for 4 h, during which time the epidermis partially separated from the exoskeleton, facilitating removal of the muscle; this phenomenon has been termed "forced apolysis" (O'Brien *et al.*, 1984). Tissues were rinsed twice in cold 0.5 *M* NaCl and 5 m*M* sodium phosphate (pH 7.4) and glycerinated in cold buffer containing 20 m*M* Tris-acetate (pH 7.5), 50% glycerol, 0.1 *M* KCl, 1 m*M* EDTA, 0.1% Triton X-100 for 2–3 h. Although fibers vary in diameter, this procedure was sufficient to extract most of the soluble proteins. Single fibers were removed from the muscle (Fig. 1) and solubilized in 0.25 or 0.5 ml SDS sample buffer [62.5 m*M* Tris-HCl (pH 6.8), 12.5% glycerol, 1.25% SDS, 1.25% β-mercaptoethanol] overnight at room temperature. Samples were heated at 90°C, 3 min before electrophoresis. Glycerination was kept to a minimum since fibers extracted for longer periods (>12 h) were not completely solubilized in SDS sample buffer.

SDS-PAGE was done using a discontinuous gel system as described (Mykles and Skinner, 1982, 1983; Mykles, 1985). BioRad low molecular weight standards were used. Gels were fixed in 10% glutaraldehyde (Schleicher and Watterson, 1983) and stained with silver (Wray *et al.*, 1981). Protein concentrations were determined by fluorescence emission spectroscopy (Mykles and Skinner, 1982).

Results and Discussion

The claw muscles of the American lobster, *Homarus americanus*, contain numerous variants of myofibrillar proteins (Mykles, 1985). These comprise two isoforms of paramyosin (P_1, M_r = 110,000; P_2, M_r = 105,000), three of troponin-T (T_1, M_r = 55,000; T_2 and T_3, M_r ~48,000), five of troponin-I (I_1, M_r = 30,000; I_2, M_r = 29,000; I_3, M_r = 28,500; I_4, M_r = 28,000; I_5, M_r = 27,000), two of troponin-C (C_1, M_r = 20,000; C_3, M_r = 17,500), three of myosin alpha light chain (αLC_1, M_r = 23,500; αLC_2 = 22,000; αLC_3 = 21,000), and one of myosin beta light chain (M_r = 18,500). The troponin-T variants T_2 and T_3 were poorly resolved on 10% polyacrylamide gels and were usually obscured by the large amount of actin (M_r = 42,000) present (Fig. 2). Neither troponin-C nor myosin αLC are stained intensely with Coomassie blue or silver; gels must be overloaded (Mykles, 1985). Gels were fixed with glutaraldehyde (Schleicher and Watterson, 1983) before silver-staining to enhance visualization of both proteins (Fig. 2).

FIGURE 2. SDS-polyacrylamide gels of glycerinated single fibers from lobster crusher (A) and cutter (B) claw closer muscles. Lane numbers correspond to locations diagrammed in Figure 1. Myofibrillar proteins are actin (*A*), myosin heavy (*HC*) and light (*LC*) chains, α-actinin (*a*), paramyosin (*P*), troponin-T (*T*), -C (*C*), and -I (*I*), and tropomyosin (*TM*). Crusher claw (A, all lanes) contained a uniform population of slow (S_1) fibers; I_4 was the major variant of troponin-I. Fast fibers (B, lanes 1–4, 6–13) contained a 75-kD protein (arrow) absent from slow fibers (B, lanes 5, 14–18). A small proportion (10–15%) of slow fibers (S_2) in the cutter claw contained variant T_1 of troponin-T (asterisk) and possessed I_2 as the major species of troponin-I (B, lane 5). Three major unidentified proteins (arrowheads) occurred in both fast and slow fibers. In S_1 fibers a minor protein slightly smaller than troponin-I_4 (A, all lanes; B, lanes 14–18) could be confused with the troponin-I_5 of fast fibers (B, lanes 1–4, 6–13); the troponin variants and the minor protein had distinct electrophoretic mobilities. Each lane contained 10 μg protein. Positions of molecular weight standards are indicated at left of (B).

In preparations from whole muscle, the closer muscles of the cutter and crusher claws differed primarily in the variants of paramyosin and troponin-I and -C (Mykles, 1985). Not surprisingly, a similar dichotomy was seen in individual fibers. Slow fibers in both claws contained one species of paramyosin (P_2), three of troponin-I, (I_1, I_2, and I_4), and one of troponin-C (C_1) (Fig. 2A, all lanes; B, lanes 5, 14–18). Individual fast fibers contained two variants of paramyosin (P_1, P_2), four of troponin-I (I_1, I_2, I_3 and I_5), and one of troponin-C (C_3) (Fig. 2B, lanes 1–4, 6–13). Slow fibers contained two small proteins (<14.5 kD) that were absent from fast fibers. In addition, fast fibers contained a 75-kD protein that was absent from slow fibers. This protein appears to be a component of the myofibrillar apparatus of fast fibers; it occurs in actomyosins extracted from cutter-claw closer and deep abdominal flexor and extensor muscles (Costello and Govind, 1984; Mykles, 1985).

Analysis of myofibrillar proteins of individual fibers has shown that the closer muscle of the crusher claw consisted entirely of slow fibers (Fig. 1A), while that of the cutter claw contained primarily fast fibers with a small population of slow fibers in the ventral and distal portions (Fig. 1B). These distributions agreed well with those obtained by morphological and histochemical methods (Lang *et al.*, 1977, 1980).

All the fibers possessed three prominent proteins (Fig. 2A, B, arrowheads; M_r = 35,000; 38,000; 49,000) that are not found in actomyosins extracted from muscle homogenates with high salt (Mykles, 1985; compare also lane *a* with lanes *b*, *c* in Fig. 3 of this paper). Since glycerination should extract all soluble proteins from the fiber, it seems likely that these are cytoskeletal elements that were not solubilized in 0.6 *M* NaCl.

It seemed possible that the various species of proteins, particularly troponin-I and paramyosin, occurred in distinct subgroups of slow and fast fibers; analysis of single fibers showed that more than one variant of a myofibrillar protein occurred in individual fibers (Fig. 2A, B). Thus the fast fibers in the cutter claw and the slow fibers in the crusher claw each consisted of a homogeneous population of fibers containing the same assemblage of myofibrillar protein variants; there were no apparent differences, either qualitative or quantitative, in the distribution of these variants.

The pattern in the slow fibers of the cutter claw, however, was more complex; two fiber types contained distinct assemblages of protein variants. Both types were slow fibers: they contained P_2 as the only species of paramyosin and they lacked the 75-kD protein characteristic of fast fibers (Fig. 2B). The majority of the slow fibers, termed S_1, had a pattern identical to that of crusher closer fibers, which contained I_4 as the major species of troponin-I (compare Fig. 2A, all lanes, with Fig. 2B, lanes 14–18). However, the minor type (S_2), comprising 10–15% of the slow fibers in the cutter claw, contained I_2 as the major variant of troponin-I and troponin-T_1, which was absent from S_1 fibers (Fig. 2B, lane 5; see also Mykles, 1985). Thus S_1 and S_2 differed both qualitatively and quantitatively only with respect to troponin variants; other myofibrillar protein variants were shared by both.

Four distinct protein assemblages have been described in lobster claw and abdominal muscles; these patterns have been classified as cutter-claw closer, crusher-claw closer, deep abdominals, and superficial abdominals (Mykles, 1985). The variant pattern of the S_2 fibers of the cutter closer is potentially a fifth assemblage. However, comparison of the S_2 assemblage with that of the superficial abdominals showed that they were similar: both shared the same myofibrillar protein variants including troponin-T_1 and I_2 as the major isoform of troponin-I (Fig. 3, compare lanes *a* and *b*). I_4, the major variant in S_1 fibers, was present in small amounts in S_2 fibers and superficial abdominal actomyosins (Fig. 3; see also Mykles, 1985). Therefore the S_2 pattern is not a new assemblage. Rather, the S_2 fibers and superficial abdominal fibers are the same fiber type with possibly similar contractile properties.

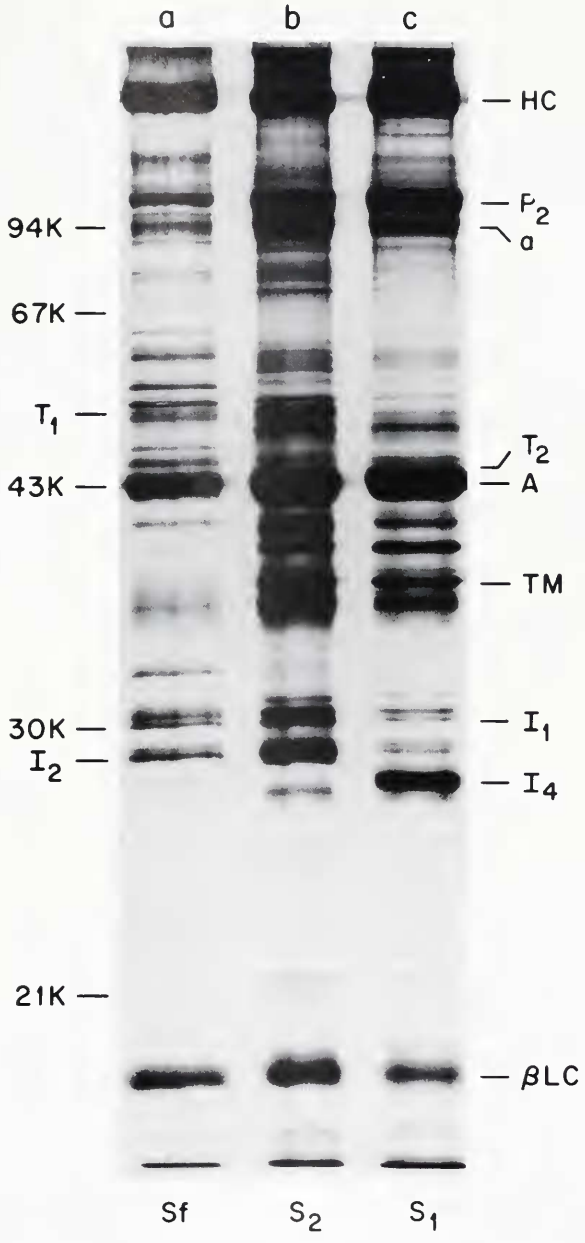

FIGURE 3. SDS-polyacrylamide gel of the two different types of slow fiber assemblages in actomyosin extracted from superficial abdominal flexor muscle (Sf, lane *a*; see Mykles, 1985) and myofibrillar proteins of glycerinated fibers from cutter closer muscle (lanes *b*, *c*). The minor type (S_2) contained a variant pattern similar to Sf muscle; both possessed troponin-T_1 and I_2 as the major variant of troponin-I. The major type (S_1) lacked T_1 and contained I_4 as the major isoform of troponin-I. Tropomyosin was originally present in Sf actomyosin (Mykles, 1985) but upon long-term (>1 year) storage in SDS sample buffer at −60°C it was not detected in later gels (lane *a*). See legend to Figure 2 for identity of myofibrillar proteins. Lane *a* contained 15 μg protein and lanes *b* and *c* each contained 20 μg protein. Positions of molecular weight standards are indicated at left.

Using histochemical methods, Kent and Govind (1981) demonstrated two types of slow fibers in the opener and closer muscles of lobster claws. The majority of the slow fibers in both claws have low ATPase and high NADH diaphorase activities, indicative of their slow contraction times and high oxidative capacity. In addition, a small group of tonic fibers found in a mid-lateral band in the distal regions of the closer muscles and in the proximal regions of the opener muscles of both claws have even lower ATPase and higher NADH diaphorase activities than the majority of slow fibers, suggesting that their contractile speeds are even slower and that the fibers are less easily fatigued.

It is not known whether the S_2 fibers described here are the same as the minor tonic fibers described by Kent and Govind. In this study the S_2 type was also found in the medial-distal region of the cutter closer. However, no S_2 fibers were found in the crusher closer even though the medial-distal region was sampled (Fig. 1A). Given the limited sampling from glycerinated muscle and the low frequency of these fibers, it is possible that the S_2 fibers were not detected. There were no apparent differences in morphology between S_1 and S_2 fibers which could be distinguished only by SDS-PAGE. An S_2 fiber was found in the ventral-proximal region of the cutter closer muscle, an area apparently lacking the lower ATPase/higher NADH diaphorase type (Kent and Govind, 1981). If the S_2 and minor tonic fibers are the same then their different contractile properties, as reflected by ATPase activity, may be related to different variants of the regulatory protein troponin or possible differences in actin and myosin variants that single-dimension gels may not detect.

Additional evidence supporting the identity of S_2 and minor tonic fibers is the similarity in the S_2 proteins to the proteins recovered in the actomyosin extracted from superficial abdominal muscles. Since the latter muscles are involved in slow movements and posture of the abdomen (Govind and Atwood, 1982), their fibers, like the minor tonic fibers, would be expected to have slow contractile speeds and to be very resistant to fatigue. It would not be surprising if they shared biochemical properties as well as myofibrillar protein variants with the S_2 fibers.

Crustacean muscle fibers have usually been characterized on the basis of physiological, histochemical, and morphological criteria. Although these methods can often discriminate between fast and slow fiber types and occasionally distinguish subtypes (Kent and Govind, 1981; Parsons and Mosse, 1982), the considerable variability makes it difficult to classify intermediate fibers. It is apparent that SDS-PAGE provides another tool for characterizing fiber types. SDS-PAGE demonstrated four polypeptides unique to fast fibers (P_1, a 75-kD protein, troponin-I_3, and -I_5) and two polypeptides unique to slow-fibers (troponin-I_4 and -C_1). The distinct variant assemblages revealed by this method can differentiate unambiguously between closely related fibers.

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